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ORIGINAL RESEARCH

Influence of Polymorphisms in the RANKL/RANK/OPG Signaling Pathway on Volumetric Bone Mineral Density and Bone Geometry at the Forearm in Men

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Abstract We sought to determine the influence of single-nucleotide polymorphisms (SNPs) in *RANKL*, *RANK*, and *OPG* on volumetric bone mineral density (vBMD) and bone geometry at the radius in men. Pairwise tag SNPs ($r^2 \geq 0.8$) for *RANKL* ($n = 8$), *RANK* ($n = 44$), and *OPG* ($n = 22$) and five SNPs near *RANKL* and *OPG* strongly associated with areal BMD in genomewide association studies were previously genotyped in men aged 40–79 years in the European Male Ageing Study (EMAS). Here, these SNPs were analyzed in a subsample of men ($n = 589$) who had peripheral quantitative computed tomography (pQCT) performed at the distal (4%) and mid-shaft (50%) radius. Estimated parameters were total and trabecular vBMD (mg/mm^3) and cross-sectional area (mm^2) at the 4% site and cortical vBMD (mg/mm^3); total, cortical, and medullary area (mm^2); cortical thickness (mm); and stress strain index (SSI) (mm^3) at the 50% site. We identified 12 *OPG* SNPs associated with vBMD and/or geometric parameters, including rs10505348 associated with total vBMD (β [95% CI] = 9.35 [2.12–16.58], $P = 0.011$), cortical vBMD (β [95% CI] = 5.62 [2.10–9.14], $P = 0.002$), cortical thickness (β [95% CI] = 0.08 [0.03–0.13], $P = 0.002$), and medullary area (β [95% CI] = −2.90 [−4.94 to −0.86], $P = 0.005$) and rs2073618 associated with cortical vBMD (β [95% CI] = −4.30 [−7.78 to −0.82], $P = 0.015$) and cortical thickness (β [95% CI] = −0.08 [−0.13 to −0.03], $P = 0.001$). Three *RANK* SNPs were associated with vBMD, including rs12956925 associated with trabecular vBMD (β [95% CI] = −7.58 [−14.01 to −1.15], $P = 0.021$). There were five *RANK* SNPs associated with geometric parameters, including rs8083511 associated with

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distal radius cross-sectional area (β [95% CI] = 8.90 [0.92–16.88], $P = 0.029$). No significant association was observed between *RANKL* SNPs and pQCT parameters. Our findings suggest that genetic variation in *OPG* and *RANK* influences radius vBMD and geometry in men.

Keywords Osteoporosis · Genetic association · Genetic polymorphism · Male · QCT

Introduction

Areal bone mineral density (BMD_a) as determined by dual-energy X-ray absorptiometry (DXA) is an important determinant of future fracture risk [1, 2]. However, other factors, including bone size and shape, also influence bone strength and susceptibility to fracture [3, 4]. Lower bone width, cortical area, and thickness as measured by DXA at both axial and peripheral skeletal sites have been associated with higher risk of fragility fractures [5]. Quantitative computed tomography (QCT) provides additional information on bone compartments, including volumetric bone mineral density (vBMD) at both predominantly cortical and trabecular sites, and geometric parameters of bone, including area, cortical thickness, and strength [6]. QCT measurements can be made at both axial and appendicular sites; the forearm is the most common appendicular site. Previous studies have shown that radius vBMD and geometry-based parameters are associated with risk of fracture in both men [7] and women [8–11].

Genetic factors are known to influence both bone mass and structure. Data from family and twin studies suggest that genetic factors explain about 50% of variation in the total radius and trabecular vBMD and up to 40% of cortical vBMD [12, 13]. In addition, a large proportion of the variation in geometric parameters such as radius cross-sectional area (27%) and cortical thickness (51%) is attributable to genetic factors [13].

The RANKL/RANK/OPG signaling pathway has a critical role in bone remodeling. Higher OPG plasma concentration has been associated with higher femoral neck BMD_a, width, and strength [14]. Strong evidence of association has previously been found between single-nucleotide polymorphisms (SNPs) within these genes and bone-related phenotypes in both candidate gene studies [15] and genomewide association studies (GWAS) [16–19]. Despite the critical importance of the RANKL/RANK/OPG signaling pathway, few studies have examined the association between

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polymorphisms within genes in this pathway and either vBMD or bone geometric parameters. Paternoster et al. [20] investigated the association between four SNPs near *RANKL*, *RANK*, and *OPG* strongly associated with lumbar spine or hip BMD_a in GWAS and tibial peripheral QCT (pQCT) measures (both density and geometry) in young European individuals. They observed significant associations between *OPG* (rs4355801 and rs6993813) and *RANK* (rs3018362) SNPs and cortical vBMD. Recently, a GWAS of pQCT outcomes at the tibia has been published by the same group. In this study, a single SNP (rs1021188) in the vicinity of *RANKL* was associated with lower cortical vBMD at the genomewide significant level and less strongly with geometric parameters. It was also associated with higher plasma levels of soluble RANKL [21]. Yamada et al. [22] also examined the association between rs2073617 and rs3134069 in *OPG* and vBMD in the distal and proximal radius in 2,220 Japanese men and women and found significant associations with radial vBMD in women. However, Yerges et al. [23, 24] found no associations between tagging and potentially functional SNPs in *RANKL*, *RANK*, and *OPG* and vBMD at the femoral neck and lumbar spine in 862 Caucasian men in the Osteoporotic Fractures in Men (MrOS) study.

Here, we used data from the European Male Ageing Study (EMAS), a population-based study of aging, to determine whether SNPs in *RANKL*, *RANK*, and *OPG* are associated with vBMD and bone geometric parameters at the mid-shaft and distal radius in middle-aged and elderly European men.

Materials and Methods

Study Participants

Men aged 40–79 years were recruited from population registers in European centres, as described before [25]. Stratified sampling was used, with the target of recruiting equal numbers of men in each of four 10-year age bands (40–49, 50–59, 60–69, and 70–79 years). Subjects were invited to participate by letter, and those who agreed were asked to attend for a more comprehensive assessment including a blood sample for genetic analysis. pQCT was performed in a subsample of subjects in two centers (Manchester, UK, and Leuven, Belgium). Participants were subsequently excluded from the analysis if they reported that at least one of their parents or grandparents was born outside Europe or North America or if they reported use of antiosteoporotic medications or systemic glucocorticoids. Ethical approval in each center was obtained in accordance with local practice and requirements, and subjects gave informed consent.

pQCT

pQCT measurements were performed in the nondominant radius using an XCT-2000 scanner (Stratec, Pforzheim, Germany) in each center following the manufacturer's standard quality-assurance procedures. Total and trabecular vBMD (mg/mm^3) and bone cross-sectional area (mm^2) were measured at the distal radius (4%, voxel size 0.4 mm). Cortical vBMD (mg/mm^3); total, cortical, and medullary area (mm^2); cortical thickness (mm); and stress strain index (SSI, mm^3) were measured at the mid-shaft radius (50%, voxel size 0.6 mm). The detailed methodology for these measurements has been described previously [26].

The European Forearm Phantom (EFP) was measured for cross-calibration between the two centers; 10 repeat measurements were taken in slices 1–4. The differences were less than precision error for total, trabecular, and cortical vBMD and for cortical area; therefore, no cross-calibration was performed between the two centers.

The short-term precision values of two repeat measurements with repositioning were as follows: total vBMD 2.1 and 1.3%; trabecular vBMD 1.27 and 1.42%; cortical vBMD 0.77 and 0.71%; and cortical area 2.4 and 1.3% in Manchester ($n = 22$) and Leuven ($n = 40$), respectively.

Genotype Data

Genotype data for pairwise tagging SNPs in *RANKL* (receptor activator of NF-κB ligand, official gene symbol *TNFSF11*) ($n = 8$), *RANK* (receptor activator of NF-κB, official gene symbol *TNFRSF11A*) ($n = 44$), and *OPG* (osteoprotegerin, official gene symbol *TNFRSF11B*) ($n = 22$) and for additional *OPG* ($n = 3$) and *RANKL* ($n = 2$) SNPs associated with BMD_a in GWAS were generated in EMAS as previously described. These SNPs were previously tested for association with markers of bone

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turnover, calcaneal quantitative ultrasound parameters, and BMD_a at the lumbar spine and total hip as measured by DXA in this cohort [15].

Statistical Analysis

Linear regression under an additive genetic model was used to test for association between SNPs and the outcome variables, adjusting for center using PLINK (1.05) [27]. Significant results were further adjusted for age, weight, and height. The results are reported as the change in the mean of the outcome variable (β coefficient) for each copy of the minor allele with 95% confidence intervals (CIs). For significant associations, the interaction between SNP and center was tested to determine if there was any between-center heterogeneity using STATA (9.2; StataCorp LP, 4905 Lakeway Drive, College Station, TX).

Assuming a 0.05 type I error, minor allele frequencies (MAFs) of 0.05–0.45, and 588 individuals, this study had >80% power to detect differences of at least 0.4 SD (MAF = 0.05) and 0.2 SD (MAF = 0.45) for all outcomes under an additive genetic model. The power of the study was also calculated assuming a more conservative type I error of 0.0008 to take into account multiple testing, which was calculated by dividing 0.05 by the number of independent genotyped SNPs according to the method described by Li and Ji [28]. With a 0.0008 type I error, the study had >80% power to detect differences of at least 0.4 SD (MAF = 0.05) and 0.3 SD (MAF = 0.45) for all outcomes. Statistical power was calculated using Quanto v1.2.3 software [29].

Results

Subject Characteristics

In total, 589 men, mean (± SD) age 60 (± 11) years, were included in the analysis. Characteristics of the pQCT parameters are presented in Table 1.

Genetic Association Analysis

RANKL

We identified four *RANKL* tag SNPs (rs346588, rs9562414, rs633137, and rs346574) associated with greater medullary area at the mid-shaft radius. However, none of these associations remained significant after adjustment for age, weight, and height (Supplementary Table 1).

There was no association between the two SNPs near *RANKL* selected from GWAS (rs9594738 and rs9594759) and pQCT outcomes.

Table 1 pQCT parameters: mean and standard deviation (SD)

Variable	*n*	Mean	SD
Distal radius			
Total vBMD (mg/mm^3)	588	399.6	71.9
Trabecular vBMD (mg/mm^3)	588	203.6	43.0
Cross-sectional area (mm^2)	588	376.0	66.7
Midshaft radius			
Cortical vBMD (mg/mm^3)	589	1,214.6	30.2
Cortical thickness (mm)	589	3.2	0.4
Total area (mm^2)	589	149.6	21.7
Cortical area (mm^2)	589	106.4	13.8
Medullary area (mm^2)	589	43.2	17.6
SSI (mm^3)	589	337.6	65.1

RANK

We identified three *RANK* SNPs associated with vBMD: rs9962159 associated with lower total vBMD, rs12956925 and rs4524035 associated with lower trabecular vBMD at the distal radius. Four *RANK* SNPs (rs9951012, rs8083511, rs6567276, and rs17665435) were associated with the distal radius cross-sectional area. A single SNP, rs12959396, was associated with both higher cortical thickness and smaller medullary area at the mid-shaft radius, but the association with medullary area did not remain significant after adjustment for age, weight, and height (Table 2). Only modest linkage disequilibrium (LD) exists between the associated SNPs, $r^2 \leq 0.36$.

There was no evidence of heterogeneity between centers for SNP associations with pQCT parameters as tested by SNP center interaction analysis.

In order to account for the number of independent SNPs tested ($n = 62$), which was calculated by the method described by Li and Ji [28], SNP associations would need to reach a P value of <0.0008 to achieve statistical significance. None of the SNPs reached this level of significance.

OPG

We identified seven tag SNPs in *OPG* associated with total and/or cortical vBMD: rs3134058 and rs3102724 associated with lower total vBMD at the distal radius; rs2073618, rs3134057, and rs1032129 associated with lower cortical vBMD at the mid-shaft radius; and rs10505348 and rs2073617 associated with both total and cortical vBMD (Table 3). There was a moderate to high LD ($r^2 > 0.50$) between most of these SNPs (Fig. 1). The SNPs rs2073618 and rs3134058 ($r^2 = 0.75$) were also associated with lower cortical thickness, and rs10505348 was associated with both higher cortical thickness and smaller medullary area at

Table 2 *RANK* (*TNFRSF11A*) SNPs associated with pQCT outcomes

SNP	Allele	MAF (%)	Type	Skeletal site	Phenotype	Adjusted for center		Adjusted for center, age, weight, and height		Associations with other bone phenotypes in EMAS [15][a]
						β (95% CI)	*P*	β (95% CI)	*P*	
rs9962159	A>G	27.5	Intronic	Distal radius	Total vBMD	−8.33 (−16.29 to −0.38)	0.041	−8.55 (−16.16 to −0.93)	0.028	–
rs12956925	G>A	16.7	Intronic	Distal radius	Trabecular vBMD	−6.79 (−13.22 to −0.36)	0.039	−7.58 (−14.01 to −1.15)	0.021	–
rs9951012	G>A	20.8	Intronic	Distal radius	Cross-sectional area	−9.28 (−17.87 to −0.69)	0.035	−10.23 (−18. 42 to −2.03)	0.015	–
rs4524035	A>G	14.3	Intronic	Distal radius	Trabecular vBMD	−8.59 (−16.30 to −0.87)	0.030	−8.35 (−16.09 to −0.60)	0.035	–
rs8083511	A>C	20.1	Intronic	Distal radius	Cross-sectional area	10.39 (2.05 to 18.73)	0.015	8.90 (0.92 to 16.88)	0.029	–
rs12959396	T>G	47.1	Intronic	Mid-shaft radius	Cortical thickness	0.05 (0.00 to 0.10)	0.046	0.06 (0.01 to 0.10)	0.026	–
				Mid-shaft radius	Medullary area	−2.31 (−4.36 to −0.26)	0.028	−1.87 (−3.85 to 0.12)	0.065	–
rs6567276	T>C	44.2	Intronic	Distal radius	Cross-sectional area	−7.50 (−14.35 to −0.65)	0.032	−8.42 (−14.97 to −1.87)	0.012	**PINP** ↑, CTX-I ↑
rs17665435	T>A	32.3	3′ downstream	Distal radius	Cross-sectional area	7.44 (0.52 to 14.36)	0.036	8.21 (1.58 to 14.83)	0.015	PINP ↓, CTX-I ↓

β the change in the mean of the outcome variable (in units e.g. mg/mm^3) for each copy of the minor allele, *CI* confidence interval, *EMAS* European Male Ageing Study, *PINP* N-terminal propeptide of procollagen I, *CTX-I* C-terminal cross-linked telopeptide of collagen type I, ↑ the SNP was associated with higher levels of the outcome, ↓ the SNP was associated with lower levels of the outcome

[a] Outcomes shown in bold were also significantly associated with the SNP in the subpopulation with pQCT

the mid-shaft radius. The tag SNP rs1032128 was only associated with lower cortical thickness at the mid-shaft radius. We also identified two tag SNPs in *OPG*, rs3102735 and rs4876868 ($r^2 = 0.65$), associated with higher SSI; but the association between rs3102735 and SSI did not remain significant after adjustment for age, weight, and height (Table 3).

All three SNPs near *OPG* selected from GWAS (rs4355801, rs6469804, and rs6993813) were associated with higher cortical vBMD at the mid-shaft radius, and rs4355801 was also associated with SSI (Table 3). No significant association was observed between these SNPs and the other pQCT outcomes.

There was no evidence of heterogeneity between centers for SNP associations with pQCT parameters as tested by SNP center interaction analysis.

None of the SNPs reached the level of significance required to correct for multiple testing ($P < 0.0008$).

Discussion

To our knowledge, this study is the first in which SNPs within *RANKL*, *RANK*, and *OPG* have been tested for association with bone vBMD and geometric parameters measured by pQCT with good gene coverage. These results are complementary to our previous analysis, which suggested that SNPs within *RANKL*, *RANK*, and *OPG* influence bone turnover and BMD_a in this population of men [15].

We found multiple SNPs in *RANK* and *OPG* associated with radius geometry or vBMD (cortical, trabecular, and total), with some SNPs associating with both. Some of these SNPs were also associated with bone turnover and BMD_a in our previous study [15].

Multiple SNPs in *RANK* in low LD were associated with total or trabecular vBMD or radius geometric parameters. Two SNPs, rs6567276 and rs17665435, which were associated with distal radius cross-sectional area, were also associated with serum levels of N-terminal propeptide of procollagen I (PINP) and C-terminal cross-linked telopeptide of collagen type I (CTX-I) bone turnover markers in this population, as reported previously [15]. The association between rs6567276 and PINP was still significant in the pQCT subpopulation. The effect on distal radius cross-sectional area was in the opposite direction to their effect on bone turnover markers [15] (Table 3).

Eight tag SNPs in *OPG* were associated with vBMD and/or cortical thickness, with some SNPs associating with both and in the same direction; e.g., rs10505348 was associated with higher vBMD (both total 4% site and cortical 50% site) and cortical thickness. Some of the *OPG* SNPs associated here with vBMD have previously been

Table 3 *OPG* (*TNFRSF11B*) SNPs associated with pQCT outcomes

SNP	Allele	MAF (%)	Type	Skeletal site	Phenotype	Adjusted for center		Adjusted for center, age, weight, and height		Associations with the other bone phenotypes in EMAS [15][a]
						β (95% CI)	*P*	β (95% CI)	*P*	
rs6993813	C>T	45.9	5′ upstream	Mid-shaft radius	Cortical vBMD	4.29 (0.72 to 7.86)	0.019	3.93 (0.47 to 7.39)	0.026	**PINP ↓, CTX-I ↓**
rs6469804	A>G	43.9	5′ upstream	Mid-shaft radius	Cortical vBMD	4.36 (0.76 to 7.95)	0.018	4.25 (0.76 to 7.73)	0.017	**PINP ↓, CTX-I ↓**
rs10505348	C>T	44.5	5′ upstream	Distal radius	Total vBMD	7.89 (0.34 to 15.44)	0.041	9.35 (2.12 to 16.58)	0.011	**PINP ↓, CTX-I ↓**
				Mid-shaft radius	Cortical vBMD	5.69 (2.08 to 9.31)	0.002	5.62 (2.10 to 9.14)	0.002	–
				Mid-shaft radius	Cortical thickness	0.06 (0.01 to 0.11)	0.026	0.08 (0.03 to 0.13)	0.002	–
				Mid-shaft radius	Medullary area	−2.33 (−4.45 to −0.21)	0.031	−2.90 (−4.94 to −0.86)	0.005	–
rs3102735	T>C	14.5	5′ upstream	Mid-shaft radius	SSI	11.23 (0.95 to 21.51)	0.033	8.83 (−0.96 to 18.61)	0.077	–
rs2073617	G>A	49.3	5′ UTR	Distal radius	Total vBMD	−9.00 (−16.27 to −1.73)	0.016	−9.82 (−16.76 to −2.88)	0.006	CTX-I ↑
				Mid-shaft radius	Cortical vBMD	−4.95 (−8.44 to −1.45)	0.006	−4.87 (−8.26 to −1.48)	0.005	–
rs2073618	G>C	47.3	Nonsynonymous	Mid-shaft radius	Cortical vBMD	−3.78 (−7.38 to −0.18)	0.040	−4.30 (−7.78 to −0.82)	0.015	**PINP ↑, CTX-I ↑**
			Lys>Asp	Mid-shaft radius	Cortical thickness	−0.07 (−0.12 to −0.01)	0.012	−0.08 (−0.13 to −0.03)	0.001	**LS BMD_a ↓**
rs3134058	G>A	40.8	Intronic	Distal radius	Total vBMD	−7.58 (−14.87 to −0.29)	0.042	−9.61 (−16.55 to −2.66)	0.007	CTX-I ↑, **LS BMD_a ↓**
				Mid-shaft radius	Cortical thickness	−0.05 (−0.10 to 0.00)	0.046	−0.06 (−0.11 to 0.01)	0.014	–
rs3134057	A>G	38.9	Intronic	Mid-shaft radius	Cortical vBMD	−3.77 (−7.37 to −0.17)	0.041	−4.21 (−7.70 to −0.73)	0.018	**CTX-I ↑, LS BMD_a ↓**
rs1032129	A>C	34.8	Intronic	Mid-shaft radius	Cortical vBMD	−4.65 (−8.33 to −0.98)	0.013	−4.20 (−7.77 to −0.63)	0.021	–
rs1032128	G>A	27.3	Intronic	Mid-shaft radius	Cortical thickness	−0.06 (−0.12 to −0.01)	0.033	−0.07 (−0.12 to −0.01)	0.016	PINP ↑
rs3102724	G>A	35.1	Intronic	Distal radius	Total vBMD	−8.00 (−15.65 to −0.35)	0.041	−10.36 (−17.65 to −3.07)	0.005	CTX-I ↑, **LS BMD_a ↓**
rs4876868	G>A	17.3	3′ downstream	Mid-shaft radius	SSI	12.20 (2.18 to 22.22)	0.017	12.48 (3.02 to 21.95)	0.010	CTX-I ↑
rs4355801	A>G	46.2	3′ downstream	Mid-shaft radius	Cortical vBMD	5.39 (1.76 to 9.02)	0.004	5.05 (1.51 to 8.59)	0.005	PINP ↓, **CTX-I ↓**
				Mid-shaft radius	SSI	−8.64 (−16.47 to −0.81)	0.031	−8.38 (−15.74 to −1.01)	0.026	–

β change in the mean of the outcome variable (in units, mg/mm^3) for each copy of the minor allele, *UTR* untranslated region, *LS* lumbar spine, ↑ SNP was associated with higher levels of the outcome, ↓ SNP was associated with lower levels of the outcome

[a] Outcomes shown in bold were also significantly associated with the SNP in the subpopulation with pQCT

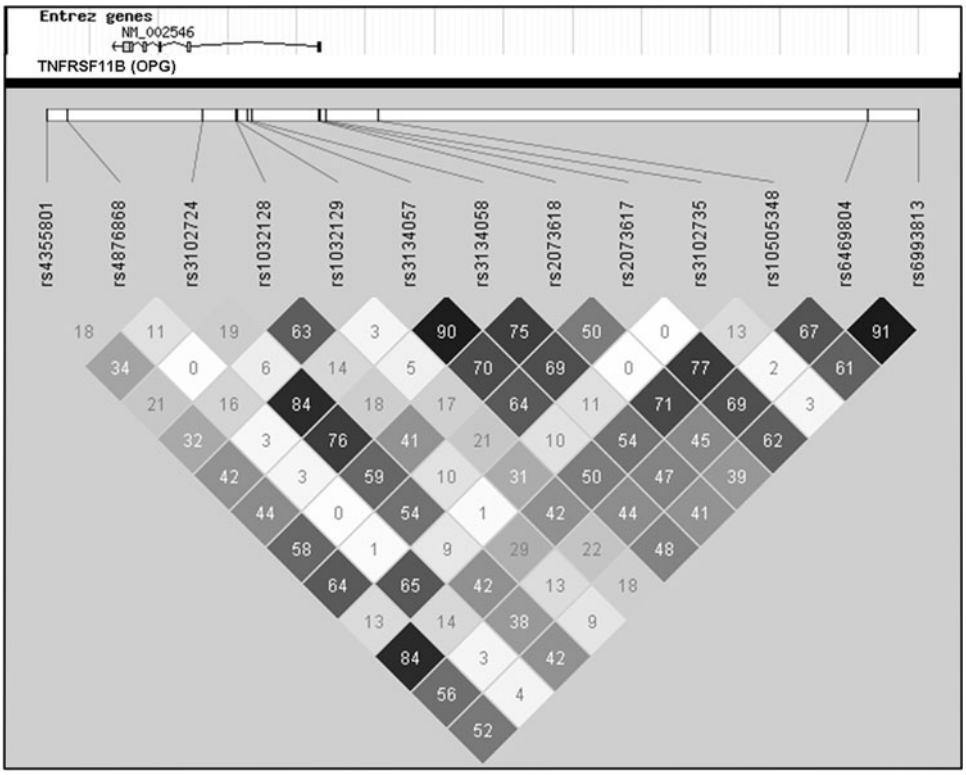

Fig. 1 The LD pattern between *OPG* (*TNFRSF11B*) SNPs associated with pQCT outcomes. SNP positions within the gene and pairwise LD (*black* $r^2 = 1$, *white* $r^2 = 0$) are shown

associated with lumbar spine BMD_a (with the same direction of effect) in a large GWAS meta-analysis at the genomewide significant level (rs10505348, rs2073617, and rs2073618) [17] and in this population as reported previously [15] (Table 3). Furthermore, rs2073617 [30, 31], rs2073618 [31–38], and rs1032129 [39] have previously been reported in association with lower BMD_a measured by DXA at different skeletal sites. As QCT measures of BMD are not confounded by bone size, this provides evidence that associations seen previously in *OPG* are truly related to density. The SNP rs2073617 has also been reported in association with lower vBMD measured by pQCT at the proximal radius in Japanese premenopausal women [22]. Most of the SNPs associated with vBMD and/or cortical thickness in our current study were also associated with bone turnover markers in this population, as previously reported [15]. A number of these associations remained significant when the analysis was limited to the subpopulation with pQCT. A lack of association with PINP or CTX-I in the subpopulation may be due to the reduced statistical power in the subpopulation; however, the effects of SNPs on bone turnover were in the opposite direction to their effects on vBMD and cortical thickness [15] (Table 3).

The association between SNPs within OPG and serum levels of OPG and soluble RANKL have been investigated in a few studies [34, 37, 40]. Jorgensen et al. [40] did not observe any significant association between rs3102735, which was associated with SSI in EMAS, and serum OPG levels. Kim et al. [37] also did not observe any significant association between rs3102735 and rs2073618 and between serum OPG and soluble RANKL. However, in the Zhao et al. study [34], the Asn-Asn genotype of rs2073618 was associated with lower serum OPG levels compared to the Lys-Lys genotype. This is consistent with our findings in which the C allele (Asn) was associated with higher bone turnover markers, lower BMD, and lower cortical thickness. OPG acts as a decoy receptor and blocks RANKL effects. RANKL increases the production, activity, and survival of osteoclasts [41]. Therefore, SNPs that decrease the activity of OPG would be expected to have a negative impact on bone health due to increased resorption.

All three SNPs near *OPG* selected from GWAS were associated with higher cortical vBMD at the mid-shaft radius. In a recent study of young (between 15 and 20 years) European individuals (1,492 males and 1,621 females from the Avon Longitudinal Study of Patents and Children [ALSPAC] and 935 males from the Gothenburg Osteoporosis and Obesity Determinants [GOOD] study), rs6993813 and rs4355801 were associated with higher cortical vBMD at the tibia [20].

Our findings contrast with those of Yerges et al. [23], who investigated SNPs in *RANKL*, *RANK*, and *OPG* and femoral neck (total, cortical, and trabecular) and lumbar spine (total) vBMD. They did not observe any significant association between *RANKL*, *RANK*, and *OPG* SNPs and

vBMD (total, cortical, or trabecular). The apparent discrepant findings may relate to different sites of assessment. In addition, their gene coverage for SNPs with MAF > 5% ranged from 1 to 100% per gene (64% on average); therefore, they might not have genotyped the associating SNPs [23, 24]. Our gene coverage for SNPs with MAF > 5% was >98%.

EMAS is a multicenter, population-based study; and standard methods were used to minimize any variation between centers. However, it has some limitations which should be considered. As the response rate was about 39%, those who declined to participate may have differed from those who agreed to participate. Therefore, caution is needed in interpreting the results, such as absolute values of the phenotype data. However, considering that the main analysis was based on a within-cohort analysis, it is unlikely that the main findings would be influenced by any selection factors. False results might be produced due to population substructure. There was no evidence of any between-center heterogeneity, and the likelihood of population stratification was minimized by excluding subjects of non-European ancestry. However, we were unable to use methods such as genomic control or principal component analysis to determine if there was any population substructure as these methods need data on a large number of SNPs. False-positive associations might be detected due to multiple testing, whereas genuine associations might be missed due to power constraints. Based on the method described by Li and Ji [28], there were 62 independent SNPs in *RANKL* ($n = 8$), *RANK* ($n = 36$), and *OPG* ($n = 18$). Using Bonferroni correction to correct for multiple testing, the new cut-off for the *P* value will be 0.0008 ($0.05/62 = 0.0008$). None of the associations we found here reached this level of significance. Some of the SNPs (i.e., in *OPG*) associated with radius vBMD in the present study have, however, previously been associated with lumbar spine BMD_a in large-scale GWASs. A technical limitation of pQCT measurement of cortical vBMD is the influence of the partial volume effect, which is due to the limited spatial resolution of pQCT. Therefore, some of the associations with cortical vBMD may be in part due to technical constraints: the thinner the cortices, the lower vBMD. We tried to minimize technical artifacts by using a higher threshold for analysis of cortical vBMD (960 mg/cm^3) rather than the traditional 710 mg/cm^3 [42], to try to reduce the partial volume effect.

To summarize, these findings add to our knowledge of the possible influence of genetic variation in the RANKL/RANK/OPG signaling pathway on bone and suggest that SNPs in *OPG* and *RANK* affect radius vBMD and geometry, especially cortical thickness, in men. The association of *OPG* is more convincing as the results are consistent with previous findings and SNPs are often associated with multiple bone-related phenotypes. However, these findings need to be validated in independent populations. If they are confirmed, fine mapping and functional studies will be needed to identify the causal variants.

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